

Formation of Intracranial Tumors by Genetically Modified Human Astrocytes Defines Four Pathways Critical in the Development of Human Anaplastic Astrocytoma¹

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Abstract

The formation of human malignant gliomas is thought to involve the accumulation of multiple genetic alterations. To define the function of specific alterations in glioma formation, we serially introduced genetic alterations functionally equivalent to those noted in human malignant gliomas into normal human astrocytes (NHAs). We then monitored the ability of each of these alterations to contribute to the growth of otherwise genetically stable NHAs into intracranial malignant gliomas. Using this model, we show that expression of human telomerase catalytic component (hTERT), but not E7-mediated inactivation of pRb or E6/E7-mediated inactivation of p53/pRb, was sufficient to initiate the tumorigenic process by circumventing cellular senescence in astrocytes. hTERT expression, even in combination with inactivation of p53/pRb, did not transform astrocytes. These alterations together, however, cooperated with ras pathway activation (initiated by expression of mutant H-Ras), but not with phosphatidylinositol 3-kinase pathway activation (initiated by expression of myristoylated Akt) or epidermal growth factor receptor activation, to allow for the formation of intracranial tumors strongly resembling p53/pRb pathway-deficient, telomerase-positive, ras-activated human grade III anaplastic astrocytomas. These results identify four pathways as key in the development of human anaplastic astrocytomas.

Introduction

Gliomas are the most common human brain tumor. Adult gliomas of astrocytic origin (astrocytomas) are divided by WHO standards into three grades, of which grade III AA³ and grade IV GBM are considered to be malignant. The malignant gliomas are believed to develop as the result of stepwise accumulations of genetic lesions (1). AAs typically exhibit loss of a functional p53 pathway (typically by p53 mutation), loss of a functional p16/pRb pathway (typically by deletion of the *p16/ARF* locus), ras pathway activation by means other than ras mutation (which is rare in any grade of glioma), and telomerase reactivation (which is rarely seen in NHA or grade II glioma; Refs. 1–4). GBMs, in addition to alterations in the p53 pathway and the p16/pRb pathway noted in AAs, also frequently amplify and express rearranged EGFR (particularly the exon 2-7 deleted, a constitutively active EGFRvIII form), and frequently contain alterations in PTEN

leading to activation of the Akt pathway (5, 6). The genetic alterations, however, are a small subset of the total changes noted in most tumors and are variable within each tumor grade. Therefore, although the unique characteristics of each astrocytoma grade are associated with a set of genetic alterations, the role these genetic changes play in glioma development remains undefined.

Although correlative approaches have given clues to the genetic lesions critical for human glioma formation, definitive identification of critical lesions and the role they play in gliomagenesis has proven difficult. Mouse models have suggested that expression of platelet-derived growth factor- β , INK4a-ARF plus EGFRvIII, and Ras plus Akt in various cell types can lead to the development of gliomas of various types and grades (7–9). It is unclear, however, how these changes put into motion the series of events that lead to gliomagenesis or how these alterations might work together with other changes commonly noted in human gliomas, *e.g.*, p53 mutation and telomerase activation, to effect transformation. As an alternative to the mouse model approach, investigators have turned to the use of normal human cells in the study of events important for transformation. Recently, it was shown that normal human fibroblasts and epithelial cells could be converted into tumor cells in a two-stage process involving immortalization by hTERT and large T antigen and transformation by oncogenic H-Ras (10, 11). In the present study, we used a similar system to identify events sufficient to immortalize and transform normal human glial cells, and to determine whether the defined genetic alterations chosen could recapitulate the human glioma phenotype.

Materials and Methods

Creation of Cell Lines. NHAs (Clonetics, Walkersville, MD) were maintained in Astrocyte Growth Medium (Clonetics) and were passaged 1:4 at 80–90% confluence with each passage considered as two PDs. To obtain retrovirus stocks, Phoenix A cells were transfected with pLXSP-puro-E7, pLXSP-puro-E6/E7, pWZL-blast-hTERT, pLRNL-neo-wtEGFR, pLRNL-neo-EGFRvIII, pLXSN-neo-H-RasV12, or pWZL-hygro-myrAkt Δ 4-129 by lipofection (12–16). Retroviral constructs were serially introduced into NHAs with selection by puromycin (0.5 μ g/ml; 4 days), blasticidin (25 μ g/ml; 4 days), neomycin (800 μ g/ml; 7 days), or hygromycin B (300 μ g/ml; 5 days) between each infection. In all cases, cultures arose from polyclonal expansion of infected cells. Two groups of E6/E7/hTERT/Ras cells were created by independent retroviral infections of E6/E7/hTERT cells with pLXSN-neo-H-RasV12. U251MG glioblastoma cells were maintained in DMEM with 10% FCS.

Preparation of Cell Extracts and Analysis by Western Blotting. Aliquots (20 μ g) of total cellular protein were separated on 7.5–12% SDS-PAGE gels and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. Western blotting was performed by standard procedures. Antigen-antibody complexes were detected using enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ). The following primary antibodies were used: p53 (DO-1); HPV16-E7 (ED17); EGFR (1005);

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³The abbreviations used are: AA, anaplastic astrocytoma; NHA, normal human astrocyte; GBM, glioblastoma multiforme; EGFR, epidermal growth factor receptor; SKY, spectral karyotyping; CGH, comparative genomic hybridization; PD, population doubling; wt, wild-type; TRAP, telomeric repeat amplification protocol; GFAP, glial fibrillary acidic protein; MAPK, mitogen-activated protein kinase.

H-Ras (F235); Akt1 (C-20); cyclin D1 (A-12) (all Santa Cruz Biotechnology, Santa Cruz, CA); and phospho-p44/42 MAPKinase (New England Biolabs, Beverly, MA).

Analysis of Telomerase Activity by TRAP. The TRAP was performed as described using the TRAPEze kit (Intergen, Gaithersburg, MD; Ref. 17). PCR products were electrophoresed on 12% polyacrylamide gels. Gels were dried and autoradiographed.

Soft Agar Assays. Cells (1×10^4) were placed in DMEM plus 10% FCS in 0.35% (w/v) low melting temperature agar between layers of 0.7% low melting temperature agar. After 3 weeks, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) and scored by counting under a microscope. U251MG cells were used as positive controls.

Tumorigenicity Assays. Immunodeficient mice (Balbc/c-*nu/nu*; Simonson, Gilroy, CA) and rats (*rnu/rnu*; Harlan, Indianapolis, IN) were maintained in pathogen-free conditions. For s.c. studies, cell suspensions (5×10^6 cells) were mixed 1:1 with Matrigel (Collaborative Biomedical Products, Franklin Lakes, NJ) and injected s.c. into anesthetized mice. Tumors were measured every 7 days, and tumor volumes were calculated using width (*a*) and length (*b*) measurements ($a^2b/2$, where $a < b$). For intracranial studies, athymic rats (Harlan) anesthetized with ketamine (60–90 mg/kg) and xylazine (7.5–10 mg/kg) were placed in a stereotaxic device using ear bars, and a hole in the skull was drilled 3 mm lateral to the bregma. Ten μ l of cell suspension (2×10^6 cells) were injected through a 25-gauge needle into the right caudate nucleus 4 mm from the bottom of the skull. The scalp was closed with surgical staples, and the animals were monitored until neurological signs became apparent.

Immunohistochemistry. Tumors were fixed in 10% formalin and embedded in paraffin. Six- μ m paraffin sections were stained with H&E. Unstained sections were deparaffinized and subjected to immunohistochemical staining. Dilution of primary antibody was 1:320 for mouse antihuman GFAP (GA5; Biogenex, San Ramon, CA). Incubation with primary antibody was overnight at 4°C. After incubation with a biotinylated secondary antibody (goat anti-mouse IgG, 1:200; Pierce Chemical, Rockford, IL) for 60 min at room temperature, antigens were revealed with horseradish peroxidase and diaminobenzidine (Vector, Burlingame, CA). Sections were counterstained in hematoxylin.

SKY Analysis. Chromosome preparations were digested with pepsin for 5 min. After washing, cells were fixed in 1% formaldehyde/50 mM MgCl₂ in PBS for 10 min. Hybridization of SKY analysis was performed according to the manufacturer's protocol (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Cells were counterstained with 4,6-diamino-2-phenylindole in antifade solution. Ten metaphase cells were examined using the Spectra Cube 200 system and Skyview analysis software (ASI).

CGH. CGH was performed according to Hirose *et al.* (18). Sample and normal DNAs were labeled with FITC-dUTP and Texas Red-dUTP (DuPont, Inc. Wilmington, DE), respectively, by nick translation using DNA polymerase I (Life Technologies, Inc.). Two hundred ng each of labeled tumor and reference DNAs and 20 μ g of human Cot-1 DNA (Life Technologies, Inc.) were denatured and hybridized to normal metaphase spreads (Vysis, Inc., Downers Grove, IL). After unhybridized probes were washed, the preparations were washed and counterstained with 4,6-diamino-2-phenylindole in antifade solution. Red, green, and blue images were acquired with a Quantitative Image Processing System (QUIPS), and the ratios of fluorescence intensity along the chromosomes were quantitated as described (18).

Results

Immortalization of Human Astrocytes. As a prerequisite to unlimited growth *in vivo*, tumor cells must first escape from controls that limit cell lifespan. The NHAs used in this study had a finite lifespan in culture of ~20 PDs. Therefore, we initially attempted to identify those events required for bypass of cellular senescence in astrocytes. NHAs at PDs 12–14 were retrovirally infected with constructs encoding hTERT and/or human papillomavirus 16 E7 (to inactivate pRb), or E6/E7 (to inactivate both p53 and pRb). The lifespans of NHAs could be extended up to 4-fold by retroviral infection with constructs encoding either E7 or E6/E7, but in all cases cells eventually ceased

Table 1 Transformation of NHA by introduction of E6/E7, hTERT, and Ras

Cell types	Colony numbers ^a	% tumor incidence (<i>n</i>) ^b
NHA	0	ND
hTERT	0	ND
E7/hTERT	0	ND
E6/E7/hTERT	0	ND
E6/E7/hTERT/wtEGFR	0	ND
E6/E7/hTERT/EGFRvIII	11 ± 4	0 (5)
E6/E7/hTERT/Akt	0	ND
E6/E7/hTERT/wtEGFR/Akt	95 ± 31	0 (5)
E6/E7/hTERT/EGFRvIII/Akt	105 ± 21	0 (5)
E6/E7/hTERT/Ras	1018 ± 205	91 (11)
E6/E7/hTERT/Ras + PD98059	105 ± 31	ND
E6/E7/Ras	0	ND
E7/hTERT/Ras	0	ND
U251MG	1455 ± 142	100 (5)

^a Cells (1×10^4) were plated in soft agar, and colonies were scored after 3 weeks. Results represent mean ± SD from two separate experiments.

^b Cells were injected s.c. into nude mice. *n*, number of animals treated. ND, not done.

proliferating. Continuous growth beyond PD 120, however, was achieved in cells expressing hTERT or a combination of E7+hTERT or E6/E7+hTERT (data not shown). Expression of E7 and in particular E6/E7 had the added effect of increasing the rate of growth of hTERT-infected cells (PD 3 and 1.5 days, respectively) which, in the absence of E6/E7, grew at a rate comparable with that of NHAs (PD 4 days; data not shown). These results suggest that although alterations in the p53 or pRb pathways can increase the growth rate and greatly extend the lifespan of NHAs, unlimited lifespan extension requires hTERT expression.

Transformation of Human Astrocytes. Although cells that have escaped from senescence can grow indefinitely in culture, anchorage-independent growth as transformed cells in soft agar and as tumors in animals often requires additional genetic alterations. To determine whether human astrocytes immortalized by various combinations of hTERT, E6, and E7 were transformed, cells were initially assessed for ability to grow in soft agar. Although positive control U251MG cells were able to grow and form colonies in soft agar, astrocytes immortalized by expression of hTERT, E7+hTERT, and E6/E7+hTERT cells were unable to grow in soft agar, and by this criteria, were not transformed (Table 1).

As potential transforming events, we considered those alterations most commonly associated with human gliomas *in vivo*. On the basis of this information, we created a series of cell lines immortalized by expression of E6/E7/hTERT and also expressing either H-Ras V12 (to mimic Ras pathway activation noted in malignant gliomas; Ref. 15), a form of Akt rendered constitutively active by truncation and fusion to a myristylation signal (to mimic Akt activation noted in GBM; Ref. 16), or the mutant form of the EGFR most commonly seen in human GBM (EGFRvIII; Ref. 6). As shown in Fig. 1, the E6/E7/hTERT cell lines all had greatly reduced levels of p53 (Fig. 1A), expressed E7 (Fig. 1B), and exhibited telomerase activity (Fig. 1F), whereas those cells additionally infected with EGFR (wt or vIII)-, H-RasV12-, or Akt-encoding constructs additionally expressed the proteins of interest (Fig. 1, C–E).

After characterization, the genetically modified cells were plated in soft agar to assess transformation and also injected s.c. into the flanks of athymic mice to assess tumor formation. As noted, the parental E6/E7/hTERT cells did not grow in soft agar and were not transformed (Table 1), suggesting that inactivation of the p53/pRb pathways, even in combination with the unlimited life span provided by hTERT expression, was insufficient to create malignant astrocytes. Two groups of E6/E7/hTERT astrocytes that were independently modified to additionally express mutant Ras did, however, form colonies in soft agar at a frequency similar to that noted for cultured

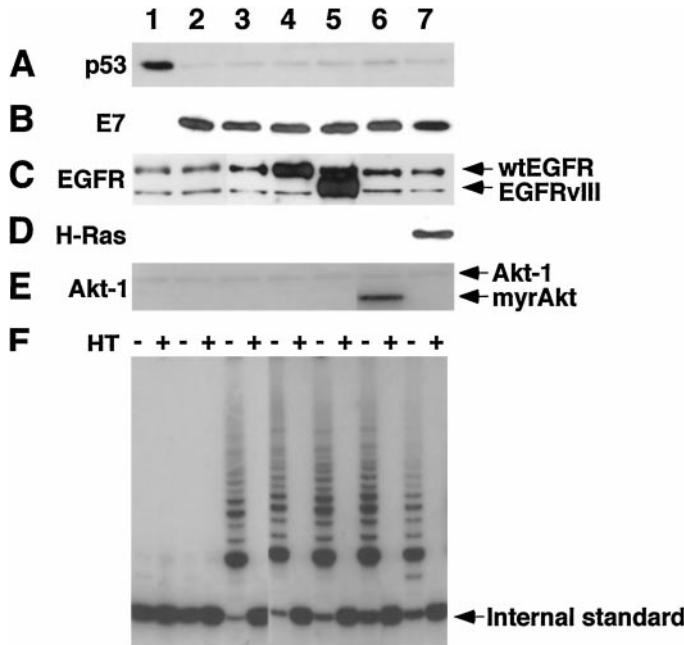


Fig. 1. Characterization of modified human astrocytes. Western blot analysis of p53 (A), E7 (B), EGFR (M_r 170,000 wt and M_r 140,000 vIII forms; C), H-Ras V12 (D), and Akt1 (M_r 56,000 wt and M_r 45,000 truncated myrAKT; E) expression in parental astrocytes (Lane 1) and in E6/E7-expressing astrocytes (Lane 2) additionally expressing hTERT (Lane 3) and wtEGFR (Lane 4), EGFRvIII (Lane 5), Akt (Lane 6), or Ras (Lane 7). F, telomerase activity in the same cells.

glioblastoma cells (U251MG; Table 1). The E6/E7/hTERT/Ras astrocytes also formed tumors in >90% of the mice into which they were injected (average size, $220 \pm 90 \text{ mm}^3$; 42 days after injection). The transforming ability of H-Ras in these cells could not be substituted by Akt, EGFR (wt or mutant), or a combination of the two. Additionally, all four events were required for cellular transformation because E7/hTERT/Ras and E6/E7/Ras cells were also unable to grow in soft agar (Table 1). These results suggest that although inactivation of the p53/pRb pathways is not sufficient for conversion of normal glia to

malignant glioma cells, inactivation of the p53/pRb pathways acts cooperatively with mutant Ras to achieve cellular transformation.

To verify that the modified glial cells injected were a polyclonal population of cells containing only the intended alterations rather than a monoclonal population of cells selected for additional genetic alterations in culture, SKY analysis was performed on 10 metaphase spreads from the E6/E7/hTERT/Ras cell population. The results of this analysis showed that 90% were diploid, and that only 2 of the 10 spreads contained detectable genetic alterations (a single unique translocation in each case; Fig. 2A). The polyclonal nature of these cells was confirmed by CGH analysis, which showed that relative to normal human cells, the E6/E7/hTERT/Ras cells contained no detectable chromosomal gains or losses (Fig. 2B). These results suggest that the genetically modified astrocytes injected were a polyclonal population that, to the best of our ability to detect, contained only the intended alterations.

Creation of Human AAs from Genetically Modified NHAs. To assess the phenotype of tumors resulting from the s.c. injection of the genetically stable E6/E7/hTERT/Ras cells, GFAP and H&E staining was performed. GFAP analysis showed that these tumors expressed GFAP and were of human glial origin (Fig. 3A). H&E staining showed that the tumors were highly cellular, contained numerous mitotic figures, and were consistent in appearance with human malignant glioma. These tumors, however, lacked areas of necrosis and vascularization (Fig. 3A) and therefore appeared not to be consistent with human GBM but rather with human AA. To verify that the AA phenotype was not an artifact of site of implantation or of the use of Matrigel in the s.c. injection, E6/E7/hTERT/Ras cells were also implanted intracranially into immunocompromised rats. As in the s.c. studies, tumors formed in 80% of the rats by 24 days after implantation (Fig. 3B). Histological examination showed that these intracranial tumors were highly cellular and contained numerous mitotic figures, again consistent with malignant human glioma. Because the intracranial gliomas lacked areas of necrosis and neovascularization, these tumors, like the s.c. tumors formed by the same cells, were also consistent in appearance with human AA. These results suggest that formation of human gliomas resembling human AAs can be accom-

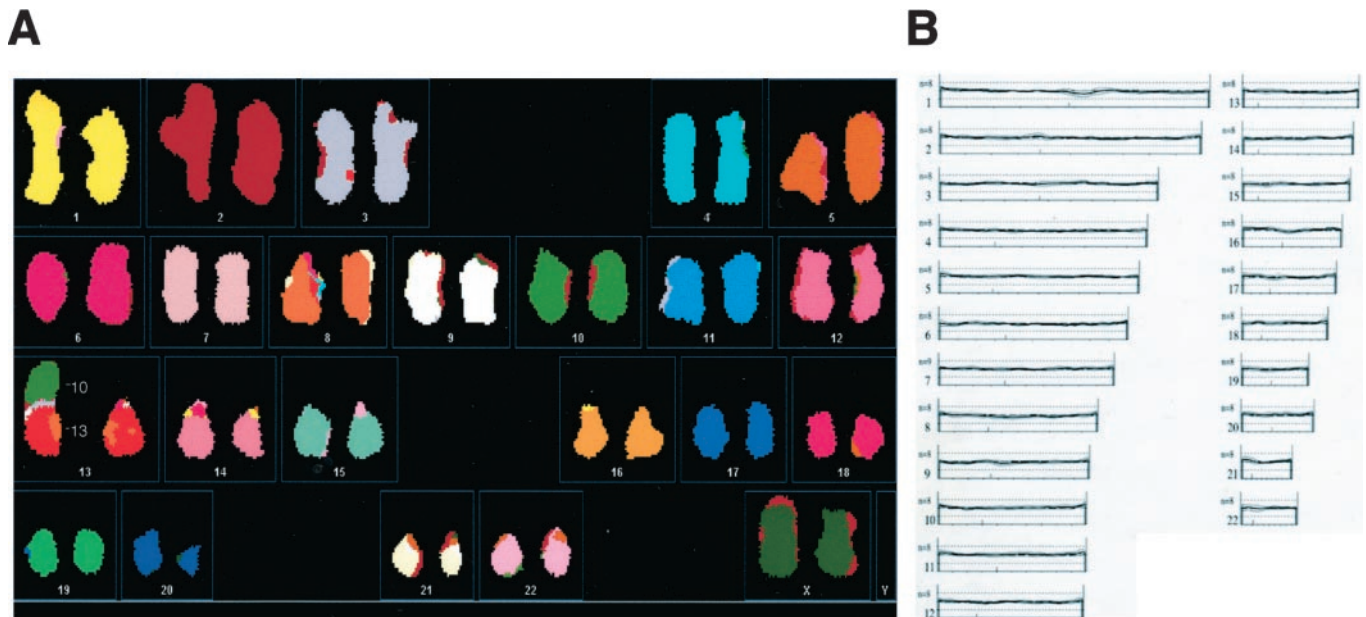


Fig. 2. SKY and CGH analysis of transformed NHA. A, spectral classified karyotype of a diploid NHA expressing E6/E7/hTERT/Ras showing a t(10;13) translocation as the only genomic rearrangement. B, CGH ratio profiles of NHAs expressing E6/E7/hTERT/Ras. X axis, the position along the chromosome (p arm to the left and q arm to the right). The centromeres are marked by a cross-hatch on the X axis. Y axis, normalized test/reference fluorescence intensity ratios.

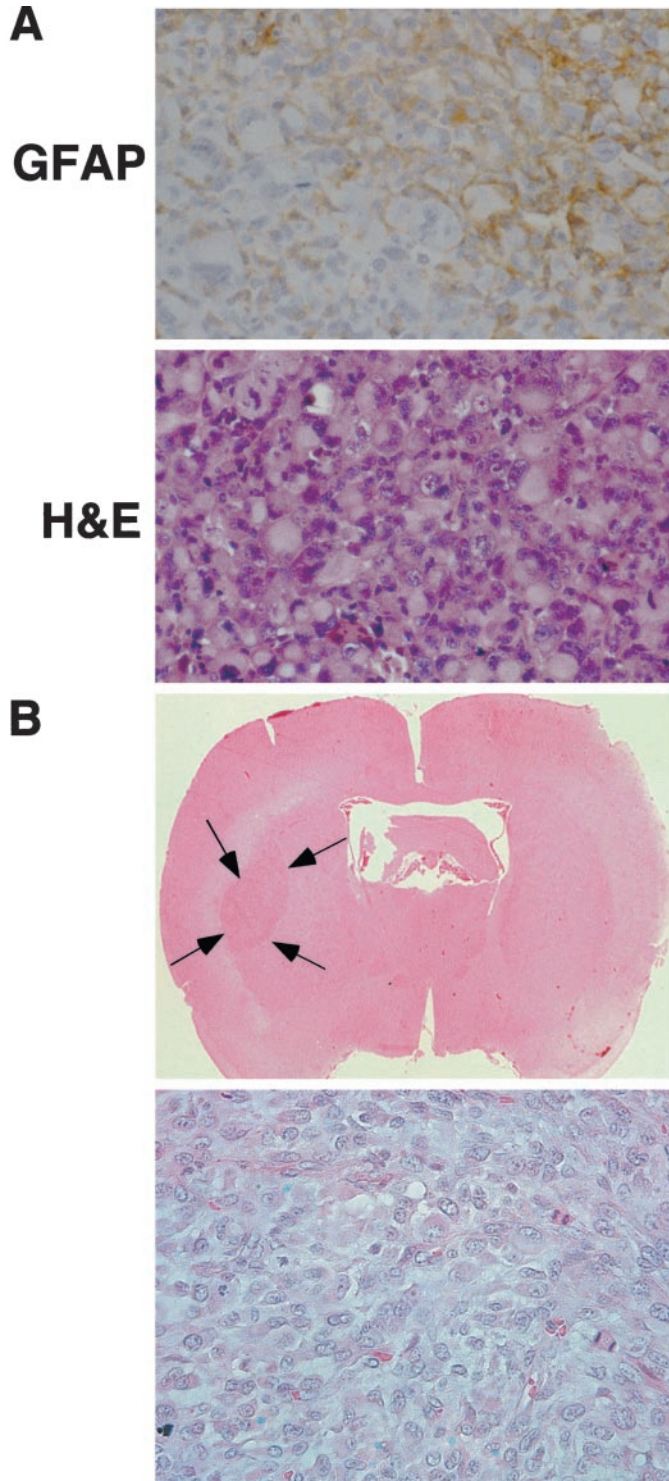


Fig. 3. Histological features of s.c. and intracranial tumors derived from NHAs expressing E6/E7, hTERT, and Ras. *A*, H&E staining and GFAP immunohistochemical analysis (with hematoxylin counterstaining) of s.c. tumors. *B*, H&E staining of intracranial tumors (gross cross-section, $\times 400$). Arrowheads mark tumor borders.

plished by four genetic alterations (hTERT expression, ras pathway activation, p53 inactivation, and pRb/p16 pathway inactivation) functionally equivalent to those found in human AAs *in vivo*.

Ras Function in Astrocytic Transformation. The transforming ability of Ras in E6/E7/hTERT/Ras astrocytes was presumed to be a result of the ability of H-Ras V12 to stimulate the downstream MAPK, phosphatidylinositol 3-kinase, and/or ral guanine nucleotide dissociation

stimulator signaling pathways (19). To better address the role of H-Ras in astrocytic transformation, levels of downstream targets of Ras were measured. E6/E7/hTERT/Ras-transformed astrocytes did not exhibit higher levels of activated phospho-MAPK than nontransformed astrocytes (E6/E7/hTERT/EGFRvIII) not expressing Ras (Fig. 4). Ras-transformed astrocytes did, however, express higher basal levels of the downstream MAPK target cyclin D1, and these basal levels were reduced by exposure of cells to PD98059 (Cell Signaling Technology, Beverly, MA), an inhibitor of MAPK kinase that blocks the signaling of Ras through the Ras-Raf-MAPK kinase-extracellular signal-regulated kinase pathway, *i.e.*, the MAPK pathway (Ref. 20; Fig. 4). Furthermore, PD98059 significantly reduced the ability of E6/E7/hTERT/Ras cells to grow in soft agar (Table 1), suggesting that the effects of Ras on the MAPK pathway were critical for Ras-induced transformation. These results suggest that although H-Ras expression in E6/E7/hTERT cells does not lead to detectably increased levels of phospho-MAPK, it does increase levels of cyclin D1 and contributes to AA formation in a PD98059-inhibitable manner.

Discussion

In the present study, we have used a human model of glioma development to define important events in human glioma development and to help explain how these alterations contribute to gliomagenesis. Of the numerous genetic alterations known to occur in gliomas *in vivo*, these studies have defined four alterations as sufficient for the formation of tumor resembling human grade III glioma.

The first key event identified in the present study in the formation of malignant gliomas was telomerase expression. The NHAs used in the present study had a finite life span and could not grow indefinitely (as would be required for tumorigenesis) unless hTERT was provided. This finding is consistent with the idea that most grade III gliomas also express telomerase and that telomerase reactivation is associated with the conversion of nonmalignant grade II gliomas to malignant grade III gliomas. The observation that hTERT alone can immortalize NHAs is consistent with the idea that most, but not all, normal human cells examined can be immortalized solely by hTERT (21). The requirement for hTERT expression in the formation of gliomas in this model also differentiates this system from transgenic mouse models of glioma, none of which take into account the activation of telomerase noted in human gliomas *in vivo*. Although the present model suggests that telomerase reactivation is an early event in glioma formation, only 20% of grade II gliomas are telomerase positive, whereas 60% are p53 deficient (2, 5). p53 mutation may, therefore, be more important than telomerase reactivation in the early stages of glioma formation *in vivo*. Early p53 inactivation in grade II gliomas may therefore provide the means for the genetic instability required for creation and selection of telomerase-positive cells, which can in turn go on to form malignant glioma. Therefore, although not necessarily the earliest event in gliomagenesis, telomerase expression does appear to be a prerequisite in the creation of malignant human gliomas.

A second key alteration important in the creation of human gliomas in the present model was functional inactivation of the p53/pRb

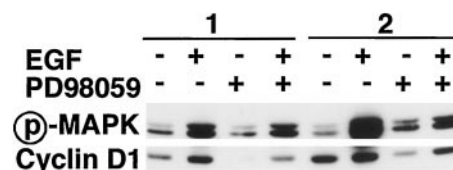


Fig. 4. Representative Western blot analysis of phospho-MAPK and cyclin D1 levels in nontransformed E6/E7/hTERT/EGFRvIII cells (1) or transformed E6/E7/hTERT/Ras cells (2) after exposure to 100 ng of EGF or 25 μM of the MEK inhibitor PD98059.

pathways by E6/E7. In the present study, E6/E7, rather than large T antigen, was used because the large T antigen used in other studies encoded both large T and small t antigens, making it less clear which protein inactivated which targets (10, 11). In the present study, inactivation of p53/pRb was not in and of itself critical, because E6/E7 expression neither immortalized nor transformed NHAs. E6/E7 expression was important, however, in the generation of malignant glioma cells in that the loss of functional p53 and pRb pathways cooperated with ras pathway activation to transform astrocytes. Loss of both pRb and p53 was critical in the present model, just as is the case in AAs in which both p53 and pRb pathways are lost in >70% of tumors (2). In AAs, however, it is unclear whether loss of the p53 pathway needs to precede loss of the pRb pathway, or whether loss of both pathways by deletion of the *p16-ARF* locus is the key event. In either case, the requirement for loss of both p53 and pRb pathways in the present model and in AAs strongly suggests that these events, perhaps by their acting cooperatively with ras pathway activation, are key in the development of malignant glioma.

The third key element in the creation of human gliomas in the present model was activation of the ras-signaling pathway. Ras-mediated activation of the MAPK pathway appeared to be important in transformation because pharmacological inhibition of this pathway by PD98059 blocked transformation *in vitro*. These results also appear to be consistent with the recent observation that in transformed mammary epithelial cells, levels of Ras expression directly correlated with tumorigenicity (11). Given these observations, however, it was puzzling that Ras-transformed astrocytes in the present study had levels of phospho-MAPK that were little different from those in nontransformed cells. One possible explanation for this finding is that at least one form of mutant Raf activates downstream MAPK effector molecules and induces cellular proliferation without significantly increasing phospho-MAPK levels (22). The results of the present study may relate to Ras-mediated activation of this or a similar pathway. Of note also is that the ability of mutant Ras to convert normal cells to cells that formed malignant gliomas could not be substituted by expression of Akt or wt or mutant EGFR, proteins the signaling pathways of which converge with the ras pathway. Clearly, the means by which Ras cooperates with p53/pRb loss to allow formation of malignant human gliomas warrants more study.

Finally, it should be noted that because the starting material for the present studies was NHAs of defined genetic composition and because the cells were transformed by expression of hTERT plus E6/E7 rather than by T antigen or passage through crisis, the cells used maintained a stable genotype. Although we cannot rule out the possibility that additional unintended genetic changes occurred, SKY and CGH analysis of cells prior to injection showed these cells to be a polyclonal population of diploid cells, few of which contained random translocations. Therefore, unlike tumors derived from other T antigen-transformed human cells (11), the gliomas generated in the present study appear, to the best of our abilities to measure, to have phenotypes dependent on the defined alterations introduced. The present system, by limiting the alterations required for glial transformation, therefore provides a powerful means of identifying factors involved in human astrocytic transformation and glioma formation.

In summary, we have shown that a limited number of defined alterations, introduced in the absence of widespread genomic changes, are sufficient to convert NHAs into cells capable of growing *s.c.* and intracranially into human gliomas of a defined phenotype. The system used in the present study should therefore be useful in identifying the factors involved in glioma formation and in clearly defining how each of the required changes contributes to gliomagenesis. The system should also prove useful in identifying additional genetic alterations that may contribute to more subtle, yet equally important, glioma characteristics.

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Yukihiko Sonoda, Tomoko Ozawa, Yuichi Hirose, et al.

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